Expression Study of Long Non-coding RNA CCAT1 in Breast Cancer Patients

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
MICROBIOLOGY

Submitted By
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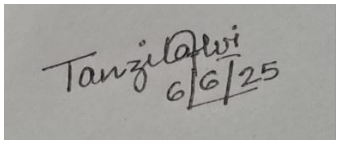

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ABBREVIATIONS

BC	Breast Cancer	**CCAT1**	Colon Cancer Associated Transcript 1
HPV	Human Papillomavirus	**HRT**	Hormone Replacement Therapy
HBV	Hepatitis B viruses	**lncRNAs**	Long non-coding RNAs
HCV	Hepatitis C Viruses	**DNA**	Deoxyribonucleic acid
MRI	Magnetic Resonance Imaging	**WHO**	World Health Organization
BBD	Benign Breast Disease	**mRNAs**	Messenger RNAs
TNBC	Triple-Negative Breast Cancer	**miRNAs**	microRNAs
DCIS	Ductal Carcinoma In-Situ	**CRC**	Colorectal Cancer
IDC	Invasive Ductal Carcinoma	**circRNAs**	Circular RNAs
ILC	Invasive Lobular Carcinoma	**snoRNAs**	Small Nucleolar RNAs
IHC	Immuno Histo Chemical	**piRNA**	Piwi-Interacting RNAs
PR	Progesterone Receptor	**mRNA**	Messenger RNAs
ER	Estrogen Receptor	**exRNAs**	Extracellular RNAs
(bCSCs)	breast Cancer Stem Cells	**ANXA2**	Annexin A2
EGFR	Epidermal Growth Factor Receptor	**EDTA**	Ethylenediaminetetraacetic Acid
BRCA1 & 2	BReast CAncer gene 1 & 2	**MCTs**	Microcentrifuge Tubes
RBC	Red Blood Cells	**PBS**	Phosphate-Buffered Saline
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	**qRT-PCR**	quantitative real-time PCR
CT value	Cycle Threshold	**CSCs**	Cancer Stem Cell-specific markers

ABSTRACT

Long non-coding RNAs (lncRNAs) have emerged as critical regulators in various biological processes, including cancer development and progression. Among them, Colon Cancer Associated Transcript 1 (CCAT1) is a well-characterized lncRNA that has been implicated in several malignancies due to its role in gene expression regulation, cellular proliferation, migration, and metastasis. The precise contribution of CCAT1 to breast cancer development and progression remains to be fully elucidated. This study aims to investigate the expression pattern of CCAT1 in breast cancer tissues and its correlation with clinic-pathological parameters in breast cancer patients to uncover its potential as blood-based predictive and prognostic biomarker.

Ten women who had been diagnosed with breast cancer and ten healthy individuals had peripheral blood samples taken for comparison. The blood was then processed, and RBC lysis buffer was used to extract the white blood cells (WBCs). Total RNA was then extracted from WBCs using the TRIzol reagent. The Qubit-4 quantification assay was then used to assess the RNA yield. The RT2 kit (QIAGEN) was then used to produce the cDNA. Then, using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) with GAPDH as an endogenous reference and CCAT1 as the target gene, the expression level of lncRNA CCAT1 in breast cancer patients was evaluated in relation to healthy controls.

The expression levels of the lncRNA CCAT1 in the circulating white blood cells (WBCs) of patients with breast cancer were significantly different from those of healthy controls, according to our findings.

The obtained data indicate that lncRNA CCAT1 expression levels may act as an informative biomarker for breast cancer diagnosis. Further investigation with a larger cohort is needed to determine the potential of lncRNA CCAT1 as a predictive and prognostic biomarker in the breast cancer patients.

KEYWORDS:

Breast cancer, Long non-coding RNA (lncRNA), CCAT1, qRT-PCR, Circulating blood-based Biomarker, Gene expression, Liquid biopsy, Cancer diagnosis

CHAPTER 1

1.1 CANCER

Cancer is a complex disease that develops from abnormal cells that grow and spread in an uncontrolled manner. Its appearance is influenced by a mix of environmental, occupational, lifestyle and genetic factors. Historical research also has shown that things such as occupational hazards, synthetic chemicals and infectious agents pose a major cancer risk **[1]**.

As the research continued, the role of infectious agents such as human papillomavirus (HPV) and hepatitis viruses (HBV and HCV) became clearer; this expanded the understanding of cancer beyond toxic exposure. Recent studies have strengthened associations between a variety of environmental and occupational exposures and specific cancers. Air pollution, for instance, has been linked to an increased risk of developing lung cancer. Exposure to pesticides has been linked to increased risks of breast and prostate and other cancers **[2]**.

Decreased exposure to known carcinogens is a proven mechanism to reduce cancer incidence, so the progressive reductions in male lung cancer were attributed to decreased tobacco usage, while the reduced incidence of bladder cancer has been attributed to decreased exposure to aromatic amines **[3]**.

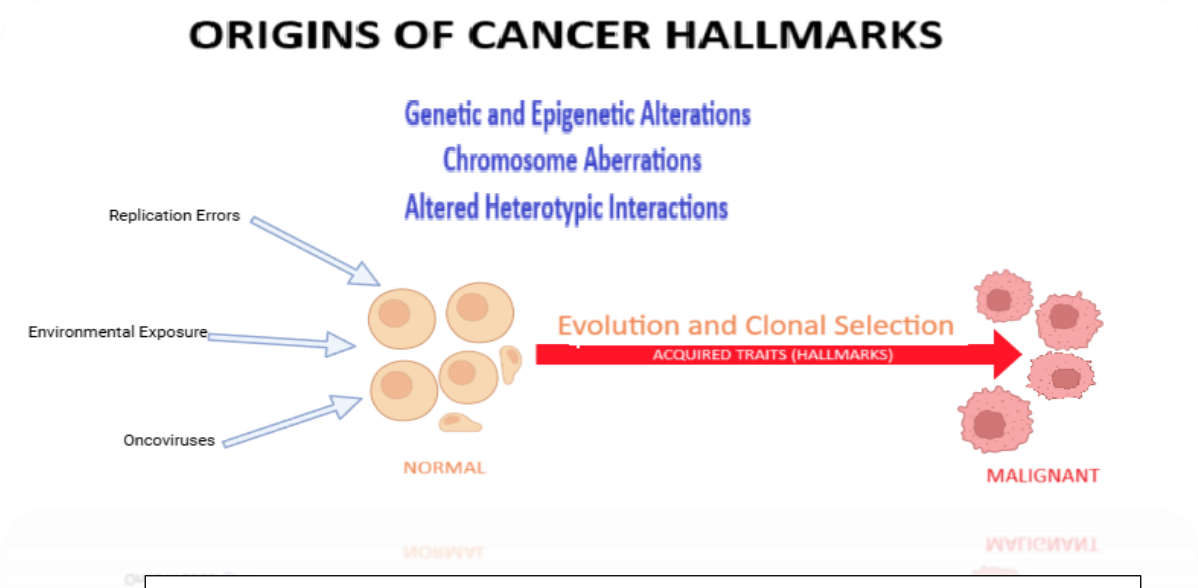

Fig 1: Origin of Cancer Hallmarks

1.2 BREAST CANCER

Breast cancer is the most common cancer among women globally, accounting for approximately 1.2 million new cases and 500,000 deaths annually. While historically more prevalent in developed nations, its incidence is rising in developing countries due to changing lifestyles, delayed diagnoses, and limited access to healthcare **[4]**.

According to 2017 statistics, breast cancer was estimated to account 30% (252,710) of all new cases of cancer among women in the United States **[4]**.

The disease primarily occurs in postmenopausal women, and a substantial proportion of cases are hormone-responsive. Hormonal dependency, as well as genetic and environmental variables, contribute to the initiation and progression of breast cancer **[5]**.

This cancer can spread to distant organs, such as the bones, liver, lungs and brain, making it more challenging to treat **[4]**.

Breast cancer screening is usually done via mammography whereas systemic therapies such as tamoxifen reduce the risk of mortality. Moreover, over the past decade, Magnetic Resonance Imaging (MRI), which provides higher sensitivity, has been used and studied more extensively **[6]**.

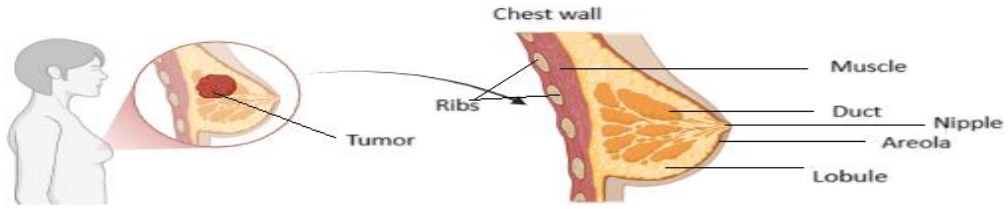

Fig 2: Breast Cancer Pathogenesis

With 1.2 million new cases and 500,000 fatalities annually, breast cancer is the most common cancer in women worldwide and continues to be a major cause of cancer-related mortality. Although more common in developed countries, such outbreaks are increasingly occurring in developing nations as well **[7]**.

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1.3 TYPES OF BREAST CANCER

Breast cancer comprises a heterogenous group of diseases, characterized by various subtypes with distinct biological and clinical features. In discussing the risk of breast cancer associated with benign breast disease (BBD), **McDivitt and colleagues'** comment that many BBDs (e.g. hyperplasia and atypia) can increase the risk of the subsequent development of malignancy substantially. Women with benign tumors called fibroadenomas are similarly at an increased risk. This study highlights the importance of monitoring benign conditions to prevent any progression **[8]**, though the study isn't designed to address subtypes such as Luminal A, Luminal B, HER2-positive or Triple-Negative breast cancer.

Colleoni and co-workers focus on luminal breast cancer subtypes, noting that Luminal A tumours, which have hormone receptors but don't overexpress HER2, frequently present with a lower proliferation rate and a better prognosis, and are often treated with endocrine therapies. In contrast, Luminal B tumors tend to be more aggressive, with the potential for HER2 amplification, and an overall worse prognosis. Their studies underscore the need for precise identification of subtypes to tailor treatments appropriately **[9]**.

HER2 overexpression defines HER2-positive breast cancer, which is aggressive but generally well -treated using drugs such as trastuzumab. On the other hand, Triple-Negative Breast Cancer (TNBC) which is characterized by the absence of estrogen, progesterone and HER2 receptors, is a highly aggressive subtype with few treatments available aside from chemotherapy. The importance of histological and molecular assessments is reiterated by **Reis-Filho and Lakhani** for an understanding of breast cancer complexity and targeted treatment determination **[10]**.

1.4 MOLECULAR SUB TYPES OF BREAST CANCER

Breast cancer can be classified into several categories, including structural-based subtypes, clinicopathological parameters, biomarkers and immune checkpoint regulators.

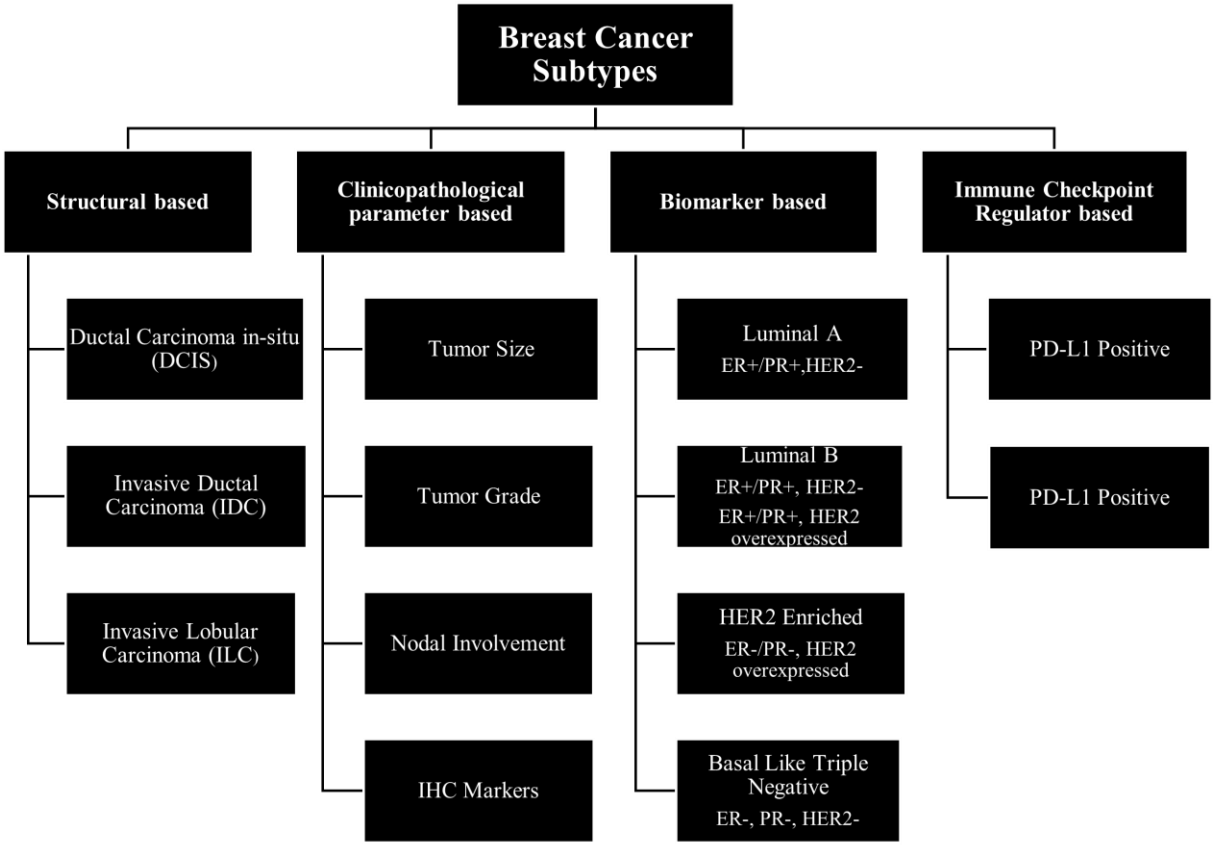

Fig 3: Sub Types of Breast Cancer

I) STRUCTURAL BASED:

i) Ductal Carcinoma In-Situ (DCIS): DCIS is a kind of breast cancer in which the aberrant cells remain inside the milk ducts rather than spreading to neighbouring tissues **[11]**.

ii) Invasive Ductal Carcinoma (IDC): The most prevalent form of breast cancer is IDC. It occurs when cancer cells begin to infiltrate the surrounding breast tissue after rupturing the duct walls. Tumor size, involvement of lymph nodes and the state of specific biomarkers are some of the variables that can affect the prognosis **[11]**.

iii) Invasive Lobular Carcinoma (ILC): ILC begins in the milk-producing lobules and has the potential to spread to neighbouring tissues. Despite being less frequent than IDC, it is frequently discovered later because of its more diffuse growth pattern, which makes detection more difficult **[12]**.

II) CLINICOPATHALOGICAL PARAMETERS-BASED:

A tumor size, grade, lymph node involvement, and immunohistochemical (IHC) markers are all considered when categorizing breast cancer. Higher grades frequently indicate more aggressive behavior, while larger tumors typically indicate more advanced stages. Understanding nodal involvement aids in determining the prognosis and the extent of the cancer's spread. Progesterone receptor (PR), estrogen receptor (ER) and HER2 status are examples of IHC indicators that are crucial for guiding therapy choices and predicting the effectiveness of treatments **[12]**.

III) HORMONE RECEPTORS-BASED:

Breast cancer subtypes include HER2-enriched, Triple-Negative, Luminal A and Luminal B biomarkers. Luminal A (ER+, PR+, HER2-) responds well to hormone therapy, develops slowly and is unlikely to recur. More aggressive and possibly HER2+, luminal B frequently necessitates a mix of hormone and HER2-targeted therapies. Although HER2-enriched (ER-, PR-, HER2+) cancer spreads swiftly, it can be successfully treated with trastuzumab and other HER2-targeted medications. Although immunotherapy is beginning to show promise, Triple-Negative (ER-, PR-, HER2-) is especially aggressive and has fewer options for targeted therapy **[13]**.

IV) IMMUNE CHEECKPOINT REGULATORS BASED:

In the fight against breast cancer, especially the more aggressive types like triple-negative breast cancer (TNBC), the immune system plays a critical role. PD-L1, an immune checkpoint protein that enables cancer cells to evade the body's immune responses, is a crucial participant in this process. Pembrolizumab and other immune checkpoint inhibitors have been shown to have a positive effect on tumors that express PD-L1. For PD-L1-positive cancers, this immunotherapy strategy holds out a lot of promise for improving treatment results and giving patients new hope **[14]**.

1.5 RISK FACTORS OF BREAST CANCER

Breast cancer is a multifaceted illness that arises from a sequence of events involving various cell types and genetic alterations. Usually beginning with ductal hyperproliferation, it can develop into tumors due to the tumor microenvironment, which includes elements such as aberrant DNA methylation patterns and inflammatory macrophages. The development of tumors, their resistance to therapy and the likelihood of recurrence are all significantly influenced by breast cancer stem cells (bCSCs). Furthermore, it is known that cancer progresses

due to mutations in key genes like EGFR, HER2, BRCA1 and BRCA2. The cancer stem cell theory, which holds that all tumor subtypes originate from the same stem cells and the stochastic theory, which contends that any breast cell can accumulate random mutations that cause cancer, are the two main theories regarding the genesis of breast cancer **[15]**.

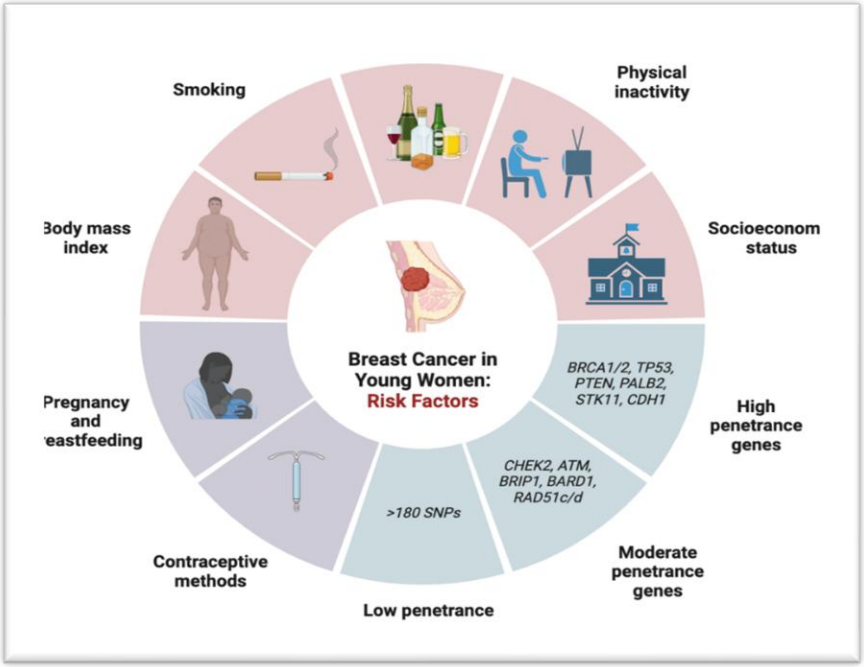

Fig 4: Risk Factors of Breast Cancer

The chance of getting breast cancer can be raised by a number of risk factors. A major contributing factor is age; women over 40 account for the majority of cases. Risk can be significantly increased by a family history of the condition, especially if the BRCA1 and BRCA2 genes are mutated. Early menstruation, a delayed menopause and a later first pregnancy are examples of reproductive factors that also come into play. Hormonal factors that increase risk include hormone replacement therapy (HRT) and increased estrogen levels from birth control pills. The risk is also increased by lifestyle choices such as smoking, eating a high-fat diet, drinking alcohol, being overweight and not exercising enough **[16]**.

To help reduce the risk of breast cancer and improve survival rates, preventive measures like genetic screening, making healthier lifestyle choices and early detection through mammograms are essential **[17].**

1.6 DIAGNOSIS

A combination of screening procedures, imaging methods, biopsies and laboratory testing is usually used to diagnose breast cancer. Although mammography is the most common screening technique, MRI and ultrasound are also useful tests, particularly for individuals who are more susceptible. To determine whether a suspicious lump is cancerous, a doctor will typically

perform a biopsy. This could be a core needle biopsy, a fine-needle aspiration or even a surgical biopsy **[18]**. The treatment plan is then shaped by laboratory tests that examine genetic mutations like BRCA1 and BRCA2, hormone receptors (like ER/PR) and HER2 status. Physicians may use bone scans, CT scans, PET scans or lymph node biopsies to determine whether the cancer has spread. Survival rates can be considerably increased by detecting it early through routine screenings and follow-ups **[19]**.

1.7 LONG NON-CODING RNA

Long non-coding RNAs or lncRNAs, are interesting RNA molecules measuring more than 200 nucleotides. Although they do not code for proteins, they perform crucial functions in gene regulation, chromatin remodelling and maintenance of diverse cellular processes. These molecules are transcribed by RNA Polymerases I, II and III and even may originate from processed introns. In contrast to their protein-coding relatives, lncRNAs are rapidly evolving, are specific to various cell types and are major contributors to cell differentiation, development and physiology **[14]**.

LncRNAs interact with chromatin-modifying complexes, which assists in shaping gene expression and genome organization. In addition, they play functions in the cytoplasm, which affects translation, metabolism and signaling pathways. Most lncRNAs possess modular structures and repeat sequences, which increase their functionality. Further, they are linked to different disease mechanisms, such as cancer development and neurological disorders **[15]**

Despite their importance to various biological functions, it can be very challenging to classify and annotate lncRNAs because they have very complex relationships with other genes. It is only going to advance in the future our knowledge about their biological functions, structural properties and therapeutic possibilities in the treatment of diseases **[16]**.

1.8 BIOMARKERS

The term "biomarker" is derived from the term "biological marker" and signifies measurable medical signs that are detectable and can be measured **(Strimbu & Tavel, 2010).** Based on the International Programme on Chemical Safety, managed by the World Health Organization (WHO) together with the United Nations and the International Labor Organization, a biomarker refers to any quantifiable substance, structure or process in the body which has the potential to influence or forecast the incidence or the consequences of disease. **[17]**. Biomarkers may be proteins such as enzymes and receptors, nucleic acids such as microRNAs and non-coding RNAs, antibodies and peptides. They may also reflect complicated biological alterations, such as gene expression and proteomic or metabolomic patterns. Since biomarkers are usually present in biological fluids such as blood (serum, plasma), excretions, and secretions (urine, stool, sputum, nipple discharge), they can be measured non-invasively and serially. However, a biopsy or imaging will be necessary to accurately assess these biomarkers **[18]**.

Hereditary genetic biomarkers can be classified as either somatic- mutations acquired in tumor DNA or germline which are inherited and detectable in DNA from sources like blood, sputum, or buccal cells. Understanding how diseases are identified, how they develop and how well therapies are working depends on these markers **[19]**.

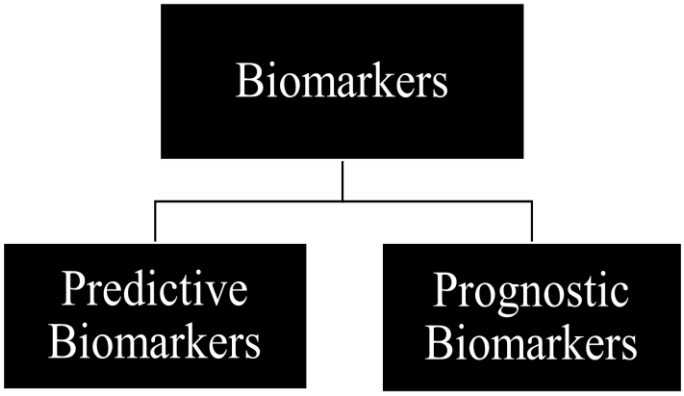

Fig 5: Types of Biomarkers

Table 1: Difference between Predictive and Prognostic Biomarkers

Features	**Predictive Biomarker**	**Prognostic Biomarker**
Definition	*Indicates the likelihood of response to a specific treatment*	*Indicates the overall disease outcome, regardless of treatment*
Purpose	*Helps guide treatment selection by identifying patients who will benefit from a particular therapy*	*Helps determine disease progression and patient survival*
Treatment Relevance	*Yes, linked to response to a therapy (e.g., targeted drugs immunotherapy)*	*No, provides information on the natural history of the disease*
Example	*HER2 overexpress ion in breast cancer predicts response to trastuzumab*	*TP53 mutation in cancer is associated with poor prognosis*
Clinical Use	*Used to personalize treatment plans*	*Used for risk stratification and prognosis estimation*

1.9 RNA BASED BIOMARKERS

RNA-derived biomarkers are increasingly popular for their significant contribution to the diagnosis, prediction and monitoring of diseases. Messenger RNAs (mRNAs) and microRNAs (miRNAs) are useful indicators in the case of colorectal cancer (CRC). Both provide useful information regarding the formation and growth of tumors. Through RNA expression patterns, researchers can identify molecular alterations linked to CRC, which can be used to detect the disease early through non-invasive techniques such as stool and blood tests **[20]**.

RNA biomarkers also have an important role in predicting the course of disease and the likelihood of recurrence, opening the door to individualized treatment strategies. For example, changes in miRNAs may influence how patients will respond to chemotherapy, particularly with fluorouracil-based therapies, guiding more effective therapy choices **[21]**.

There remain, however, some challenges to be overcome, like genetic heterogeneity, problems in RNA stability and the need for standardized protocols **[23]**. Circulating tumor RNAs and stool-based miRNAs notwithstanding these challenges are very promising in increasing CRC screening.

One such region of special concern is the long non-coding RNA (lncRNA) CCAT1, which may act as a non-invasive biomarker associated with CRC development, gene expression and oncogenic pathways. CCAT1 has implications beyond CRC, being implicated in gastric, liver and breast cancers highlighting its role as a target for future therapies **[24]**.

1.10 TYPES OF RNA BASED BIOMARKERS

i) MicroRNAs (miRNAs) are small, non-coding RNAs that are involved in an important regulation of gene expression post-transcriptionally by binding to the 3' UTR of target mRNA, modulating processes such as proliferation of cells, differentiation and apoptosis as cancer activating or cancer blocking genes **[25]**.

ii) Long Non-Coding RNAs (lncRNAs), more than 200 nucleotides, and play significant roles in the regulation of genes and remodeling chromatin. Certain lncRNAs, such as CRCAL-3 and CRCAL-4, were discovered to be dysregulated in colorectal cancer (CRC) and influence the regulation of the cell cycle **[20]**.

iii) Circular RNAs (circRNAs) stable RNAs form covalently closed loops and acts as miRNA sponges, managing gene transcription and assisting protein translation **[26]**.

iv) Small Nucleolar RNAs (snoRNAs) are also significant; they are involved in the maturation of ribosomal RNA and are associated with processes such as alternative splicing and silencing of genes **[27]**.

v) Piwi-Interacting RNAs (piRNAs) are short RNAs associate with Piwi proteins and participate in silencing transposons, cell proliferation and cancer development **[28]**.

vi) Messenger RNAs (mRNAs) are of prime type and their expression patterns have the potential to give important clues to disease pathology. PAM50 and other multi-gene panels are used to categorize and forecast the prognosis of breast cancer **[27]**.

vii) Extracellular RNAs (exRNAs), which are present in biofluids like saliva and serum. These are promising non-invasive biomarkers since they include miRNAs and circRNAs and are frequently maintained by vesicle structures or RNA-binding proteins **[28]**.

1.11 CCAT1 AND ITS ROLE IN BREAST CANCER

Long non-coding RNA CCAT1 has a significant role in the progression, metastasis, and prognosis of breast cancer. Studies suggest that CCAT1 is strongly upregulated in breast cancer tissues, particularly in breast cancer stem cells (BCSCs) and is associated with worse prognosis for patients **[29]**.

CCAT1 promotes cancer cell proliferation, invasion and migration through the activation of the WNT/β-catenin signaling pathway. TCF4 is upregulated and β-catenin is translocated to the nucleus as a result of interaction with Annexin A2 (ANXA2) and certain microRNAs (miR-204/211 and miR-148a/152). This activation augments cancer stemness and this is implicated in tumor metastasis and recurrence **[30]**.

For triple-negative breast cancer (TNBC), CCAT1 promotes tumor development through suppressing miR-218/ZFX signaling. If CCAT1 is knocked down, there's upregulation of miR-218 that suppresses the ZFX oncogene, thereby reducing TNBC cell growth, migration and invasion. This indicates that CCAT1 functions as an oncogene in TNBC by targeting pathways that typically inhibit tumors **[31]**.

Furthermore, CCAT1 also presents as a promising prognostic biomarker. Its overexpression is associated with increased tumor stages, lymph node metastases and poorer survival rates in breast cancer patients **[32].** Due to its primary role in the progression of cancer, CCAT1 may prove to be an effective therapeutic target against breast cancer **[33]**.

CHAPTER 2

MATERIALS & METHODOLOGY

BLOOD PROCESSING

2.1 Sample: **Ten** breast cancer patients' peripheral blood samples and **ten** healthy control peripheral blood samples were obtained. **(Table 2)**

2.2 Equipment & Instruments

- Pipettes & Pipette Tips (for precise liquid handling)
- Vortex Mixer (for homogenization)
- Microcentrifuge Tubes (MCTs) (sterile, for sample collection)
- Applied Biosystems thermocycler,
- Nanodrop Lite
- PCR hood
- CFX96 RT-qPCR
- Eppendorf centrifuge (capable of cooling to 4°C and high-speed spins)
- Vortex (for homogenization)
- Heating block
- pH meter
- Micropipettes

2.3 Reagents & Buffers

- RBC Lysis Buffer (for removal of red blood cell)
- Phosphate-Buffered Saline (PBS) (for washing the pellet)
- TRIZOL Reagent (for nucleic acid preservation)
- Sigma analytical grade chloroform
- Sigma analytical grade isopropanol
- 75% ethanol
- NFH20
- 5x QuantiScriptRT Buffer
- RT Primer Mix,
- gDNA Wipeout Buffer 7x
- Quantiscript Reverse Transcriptase
- 2x QuantiTect SYBR Green
- Forward and Reverse primers for MALAT1
- Forward and Reverse primers for GAPDH

2.4 Miscellaneous:

- EDTA Tubes
- PCR tubes
- MCTs
- Cryogenic Storage (-80°C Freezer) (for long-term preservation of sample)

- Labels & Markers (for proper sample identification)

Table 2: Clinicopathological information of the patients incorporated

PATIENTS	DIAGNOSIS	AGE	ER	PR	HER2	NODAL INVOLVEMENT	NO. OF CHEMOTHERAPY CYCLES
PATIENT 1	Invasive Ductal Carcinoma	54	+	+	-	**Pt3Na**	6 cycles
PATIENT 2	Residual Viable Infiltrating Ductal Carcinoma	40	+	-	-	**yT3N2**	6 cycles
PATIENT 3	Invasive Ductal Carcinoma	46	+	+	+	**ypT1sN0**	8 cycles
PATIENT 4	Invasive Breast Carcinoma	43	+	+	-	**ypT1N0**	8 cycles
PATIENT 5	Infiltrating Ductal Carcinoma	61	+	+	-	**pT2N1**	8 cycles
PATIENT 6	Invasive Carcinoma	51	+	+	+	**ypT3N0**	6 cycles
PATIENT 7	Invasive Breast Carcinoma	35	+	+	-	**ypT0N0**	4 cycles
PATIENT 8	Invasive Carcinoma	51	-	-	- (Eq.)	**ypT2N0**	4 cycles
PATIENT 9	Invasive Ductal Carcinoma	53	+	+	-	**ypT2N1a**	4 cycles
PATIENT 10	Residual Invasive Mammary Carcinoma	53	+	+	+	**ypT1a(m)N0**	6 cycles

2.5 WBC ISOLATION PROTOCOL

This protocol is performed to efficiently separate and preserve different blood components for downstream molecular analysis. Plasma is isolated for biochemical and proteomic studies, while cellular components are processed for nucleic acid extraction. The use of RBC lysis

buffer, PBS washing and TRIzol LS reagent ensures high-quality isolation of nucleic acids for genetic and transcriptomic research.

Blood processing for Breast Carcinoma patients and healthy controls:

A. Blood samples were collected from ten breast carcinoma patients and ten healthy controls in EDTA Tubes.

B. The blood samples were centrifuged at 2000g for 10 minutes at 4°C. After centrifugation, the components of blood (Red Blood Cells, White Blood Cells and platelets) precipitate to the bottom while the plasma remains at the top of the EDTA tubes.

C. The plasma was then separated carefully from the EDTA Tubes by pipetting. This whole process was conducted on ice to prevent the degradation of RNAs present in the samples.

D. After the separation of plasma, RBC lysis buffer was added to the EDTA tubes and incubated for 20 minutes. RBC lysis buffer is effective for lysing nucleated red blood cells.

E. The samples were then centrifuged at 3500g for 15 minutes. After centrifugation, WBCs precipitate at the bottom of the EDTA Tubes.

F. After discarding the supernatant, WBCs were washed with RBC lysis buffer 2-3 times to eliminate the presence of extra RBCs in the samples.

Plasma was put into sterile MCTs → Stored at -80°C.

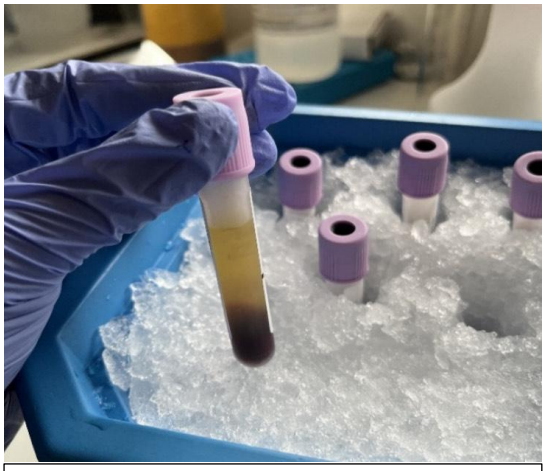

Fig 6: Blood plasma separated by centrifugation from blood sample from Breast Cancer patient

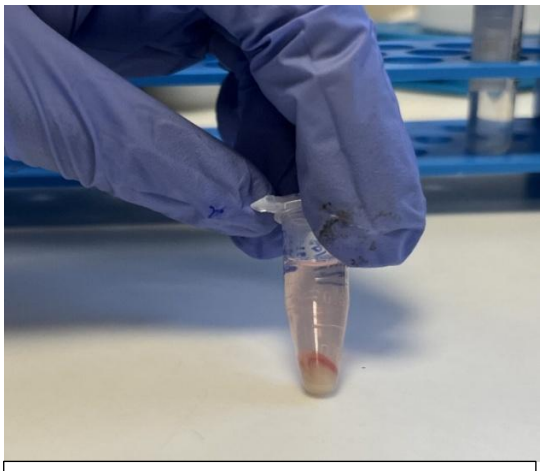

Fig 7: The white-coloured WBC pellet was isolated after RBC lysis using RBC lysis buffer and then stored at -80 °C.

RNA extraction from WBCs by TRIzol reagent:

TRIzol reagent was used to extract RNA from the obtained WBCs

A. White Blood Cells obtained from blood processing were suspended in 1 ml of TRIzol.

B. For lysis, 0.2 ml chloroform was added per 1 ml of TRIzol Reagent, then the mixture was thoroughly homogenized by pipetting up and down.

C. Incubated for 10-15 minutes**.**

D. The sample was centrifuged for 30 minutes at 12,000×g at 4°C. The combination separates into a colorless upper aqueous phase, an interphase and a lower red phenol-chloroform.

E. The aqueous phase containing the RNA was transferred to a new tube by angling the tube at 45° and pipetting the solution out.

F. For lysis, 0.5 ml of isopropanol was added to the aqueous phase per 1 ml of TRIzol Reagent.

G. Incubated for 1 hour at 4° C.

H. The sample was centrifuged for 30 minutes at 12000×g at 4° C. Total RNA precipitate formed a white gel-like pellet at the bottom of the tube.

I. The supernatant was discarded with a micropipette.

J. The pellet was resuspended in 1 ml of 75% ethanol per 1 ml of TRIzol Reagent.

K. The sample was vortexed before being centrifuged at 4° C for 5 minutes at 7500g.

L. The supernatant was discarded by using a micropipette.

M. The RNA pellet was dried in air or vacuum for 5-10 minutes.

N. The pellet was resuspended in 20-50 µl of RNase-free water (warm) by pipetting up and down.

O. Incubated in a water bath or heat block at 55-60°C for 10-15 minutes.

P. Total RNA was measured at nanodrop/qubit

Q. Storage for Future Analysis at -80°C

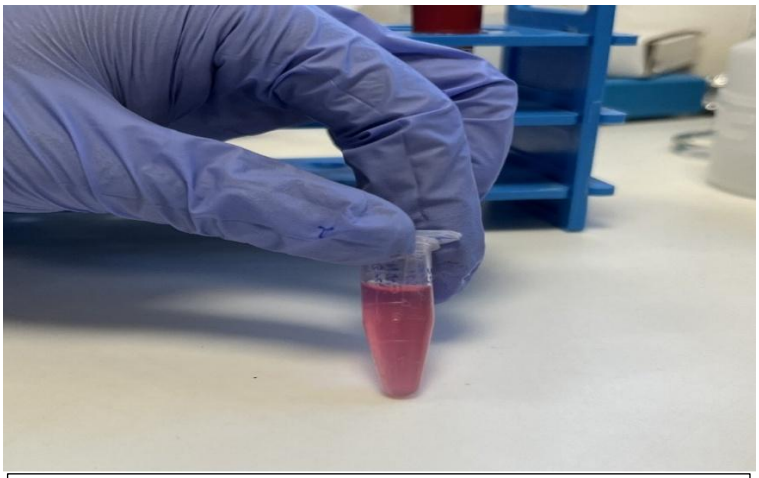

Fig 8: RNA extraction from WBC sample was performed using TRIzol reagent

2.6 RNA Quantification

STEP1:
Qubit Fluorometer was used with RNA High Sensitivity Kit
↓
If RNA concentration was low → Concentrated with vacuum concentrator
↓
Re-quantify RNA was done using Qubit 4/nanodrop

cDNA Preparation with QuantiTect Reverse Transcription:

A. The Quantitect RT Kit was used to make the cDNA.
B. Place the template RNA on ice to thaw.
C. Prepare the genomic DNA elimination reaction on ice according to **Table 3**

TABLE 3: cDNA Reaction Master Mix

Change required	10 Healthy Control's sample	10 Breast Cancer patients' samples (µL)	Total (µL)
Quantiscript Reverse Transcription	**1x2 = 2**	**1x2 = 2**	**4**
RT Buffer 5X	**4x2 = 8**	**4x2 = 8**	**16**
RT Primer mix	**1x2 = 2**	**1x2 = 2**	**4**
Total Volume	**12**	**12**	**24**

D. Incubate for 2 min at 42℃. Then place immediately on ice.

E. Prepare the reverse-transcription reaction master mix according to the manufacturer's instructions **(Table 4).**

TABLE 4: RT-qPCR Reaction Mix for CCAT1

Change Required	CCAT1	GAPDH
	4X2 (Duplicate)+1NTC = 9 wells	**4X2 (Duplicate)+1NTC = 9 wells**
SYBR Green	**5x9 = 45**	**5x9 = 45**
Forward Primer	**1x9 = 9**	**1x9 = 9**
Reverse Primer	**1x9 = 9**	**1x9 = 9**
NF-H20	**2x9 = 18**	**2x9 = 18**
Total	**9 µL /well**	**9 µL /well**

F. Add 1µg of RNA to each 20 µl of Reverse Transcriptase-PCR reaction

G. Incubate the mixture at 42℃ for 15 minutes.

H. Incubate the mixture at 95℃ for 3 minutes to inactivate Quantiscript Reverse Transcriptase

I. Quantitative real-time PCR.

2.5 Real-Time PCR (qPCR)

After cDNA preparation, the Quantitect SYBR Green PCR kit was used to perform RT-qPCR. The following are the steps involved in the procedure:

A. Make a reaction mix according to the instructions **(Table 5).**

Table 5: RT-qPCR reaction mix for MALAT1

	MALAT1	**GAPDH**
	21×2(Duplicate)+1 NTC=43 wells	**21×2(Duplicate)+1 NTC= 43 wells**
SYBR Green	**5×43 = 215**	**5×43= 215**
Forward Primer	**1×43= 43**	**1×43= 43**
Reverse Primer	**1×43= 43**	**1×43= 43**
NF-H20	**2×43= 86**	**2×43= 86**
Total	**9µl/well**	**9µl/well**

B. Fill each well with the reaction mix.

C. Add 1µl template cDNA (10ng) in each well and 1µl NFH_2O for the Non-template Controls (NTCs).

D. Set up the real-time cycler in Figure 10.

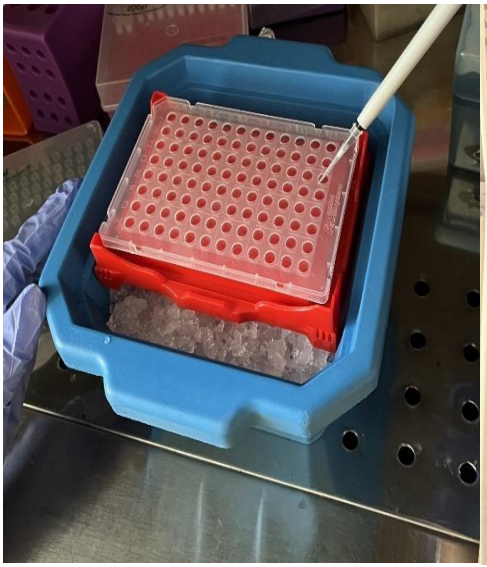

Fig 9: RT-qPCR reaction mixes plating into 96 wells

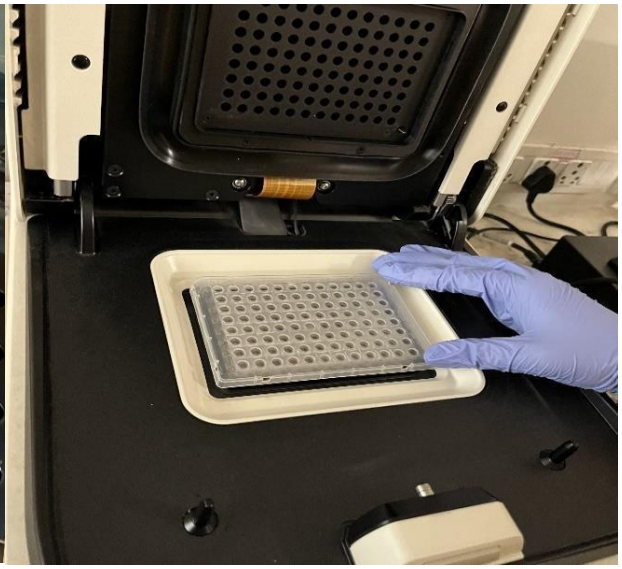

Fig 10: RT-qPCR machine

Fig 11: Real-time PCR cycle

E. The target gene's relative expression levels were measured using the endogenous house-keeping gene GAPDH as an internal control. qPCR for **CCAT1** was performed using **GAPDH** as endogenous control. qPCR was done using SYBR Green or probe-based master mix. Relative quantification of CCAT1 was calculated using **ΔΔCt method**.

CHAPTER 3

3.1 qRT-PCR RESULTS

Quantitative Real Time-PCR analysis revealed that the expression of lncRNA CCAT1 decreases significantly in the white blood cells (WBCs) of the eleven Breast cancer patients compared to that of the ten healthy control samples. The red colour curves are for endogenous control (GAPDH) and the blue colour curves are for lncRNA CCAT1.

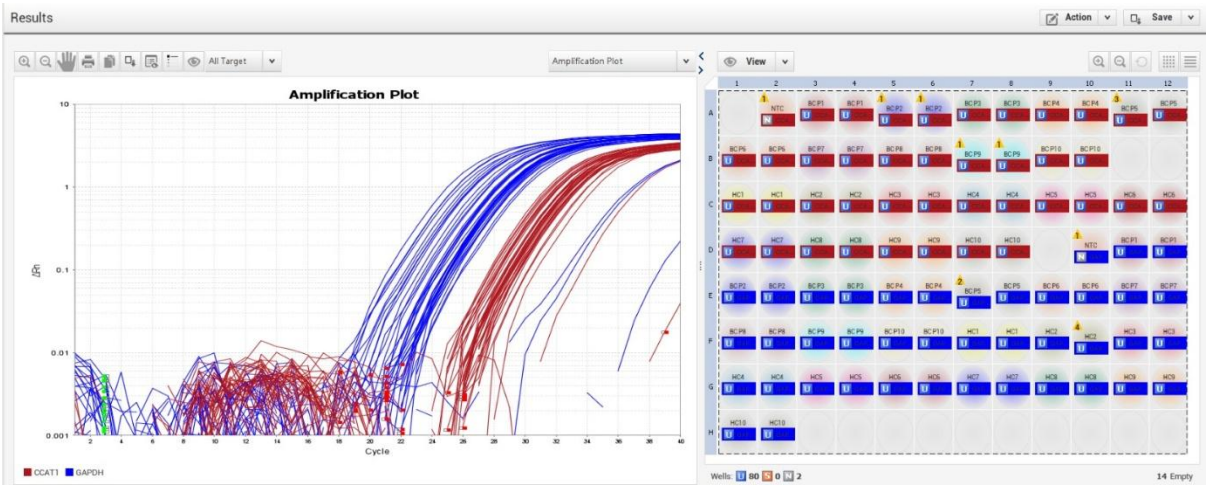

Fig 12: qPCR Amplification

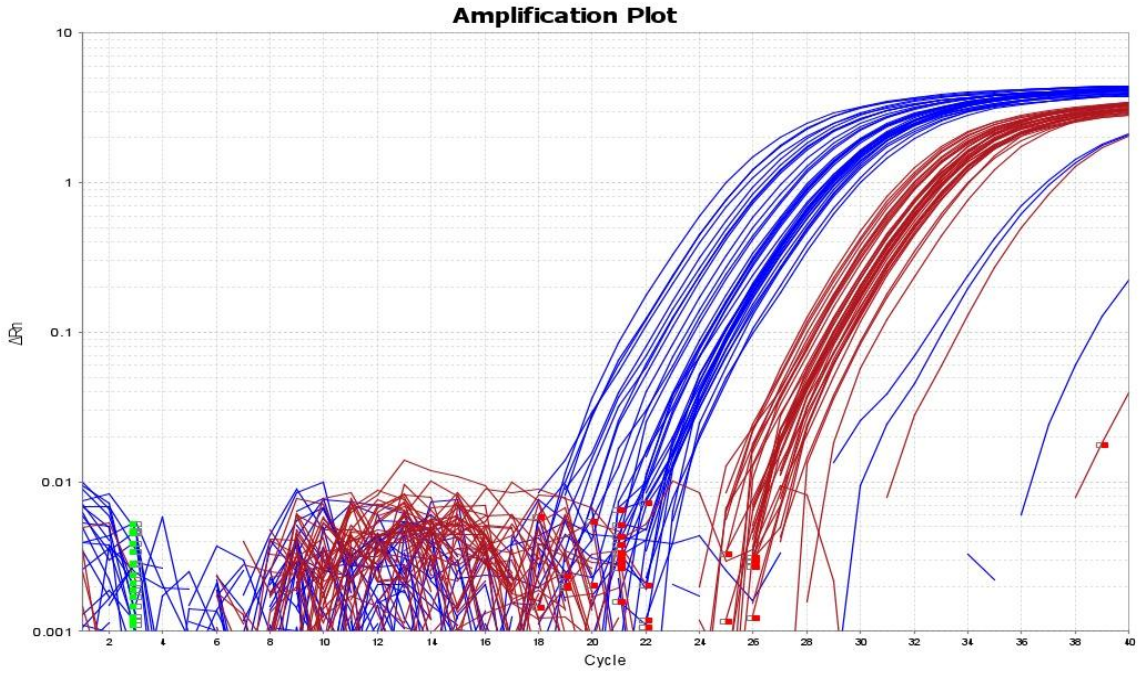

Fig 13: Overview for CCAT1 and GAPDH Expression Analysis

CT values:

The CT value is inversely proportional to the starting load of the cDNA. Hence the CT values were obtained from the RT-qPCR analysis, as depicted in **Table 6**.

TABLE 6: CT VALUES

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
1	Sample name	Target gene	CT1	CT2	Average		Internal control	CT1	CT2	Average		ΔCT	ΔΔCT		2^-ΔΔCT			
2	Sample 1	CCAT1	30.27	30.66	30.46		GAPDH	25.94	26.01	25.97		4.49	-0.92		1.892			
3	Sample 2	CCAT1	31.73	31.02	31.38		GAPDH	27.86	27.69	27.77		3.60	-1.81		3.504			
4	Sample 3	CCAT1	30.79	30.59	30.69		GAPDH	27.25	27.13	27.19		3.50	-1.91		3.750			
5	Sample 4	CCAT1	30.40	30.62	30.51		GAPDH	26.22	26.08	26.15		4.36	-1.05		2.070			
6	Sample 5	CCAT1		35.12	35.12		GAPDH	-	34.78	34.78		0.34	-5.07		33.591		Not considered	
7	Sample 6	CCAT1	30.75	30.60	30.67		GAPDH	26.69	26.63	26.66		4.01	-1.40		2.631			
8	Sample 7	CCAT1	29.22	29.46	29.34		GAPDH	26.74	26.96	26.85		2.49	-2.92		7.571			
9	Sample 8	CCAT1	31.04	31.11	31.08		GAPDH	26.77	26.72	26.75		4.33	-1.08		2.116			
10	Sample 9	CCAT1	30.39	31.32	30.86		GAPDH	26.88	26.99	26.93		3.92	-1.49		2.807			
11	Sample 10	CCAT1	29.95	30.02	29.99		GAPDH	26.81	26.88	26.84		3.14	-2.27		4.815			
12	Sample 11 HC	CCAT1	29.80	29.38	29.59		GAPDH	23.45	23.46	23.46		6.13	0.72		0.606			
13	Sample 12 HC	CCAT1	30.82	30.31	30.57		GAPDH	24.04	_	24.04		6.53	1.12		0.461			
14	Sample 13 HC	CCAT1	31.26	31.22	31.24		GAPDH	24.27	24.45	24.36		6.88	1.47		0.361			
15	Sample 14 HC	CCAT1	32.34	31.87	32.10		GAPDH	26.17	26.07	26.12		5.98	0.57		0.673			
16	Sample 15 HC	CCAT1	30.95	30.58	30.76		GAPDH	25.56	25.46	25.51		5.25	-0.16		1.116			
17	Sample 16 HC	CCAT1	30.38	30.85	30.62		GAPDH	24.57	24.45	24.51		6.10	0.69		0.618			
18	Sample 17 HC	CCAT1	29.79	29.75	29.77		GAPDH	24.98	25.26	25.12		4.65	-0.76		1.694			
19	Sample 18 HC	CCAT1	30.77	30.68	30.73		GAPDH	24.98	25.26	25.12		5.60	0.19		0.874			
20	Sample 19 HC	CCAT1	31.10	31.14	31.12		GAPDH	27.33	27.26	27.30		3.82	-1.59		3.008			
21	Sample 20 HC	CCAT1	29.59	29.88	29.74		GAPDH	26.47	26.71	26.59		3.15	-2.26		4.801			
22																		
23												Average ΔCT of healthy control						
24												5.410419						

Circulating CCAT1is overexpressed in Breast Cancer patients compared to the group of healthy control

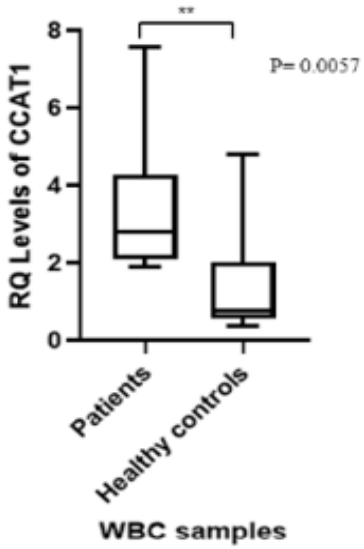

Fig 14: Relative quantification of circulating CCAT1 expression levels in Breast Cancer patients vs. healthy control group shows circulating CCAT1 levels are significantly higher in Breast Cancer patients

The expression levels of lnRNA CCAT1 in the peripheral blood of the 10 breast cancer patients and 10 healthy individuals as control was analysed by performing RT-PCR. It was observed in the study that the circulating lnRNA CCAT1 is overexpressed in samples of breast cancer patients when compared to normal healthy controls. Through the statistical analysis it was found that significantly (**P = 0.0057**) circulating CCAT1 levels are upregulated in BC patients.

Circulating CCAT1 is upregulated in HER2- positive patients as compared to HER2-negative patients

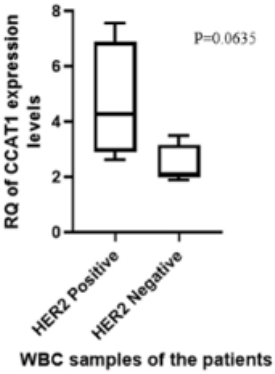

Fig 15: Relative quantification of circulating CCAT1 expression levels in HER2 Positive patients vs. HER2 Negative patients shows circulating CCAT1 levels are significantly higher in Breast Cancer patients.

Further, it was concluded that circulating CCAT1 expression levels has the potential to be used as biomarkers to identify HER-2 positive and HER-2 negative breast cancer patients. After performing RT-PCR and calculating the relative quantification, the statistical analysis was performed, which showed the result that was not statistically significant (**P = 0.0635**). The statistical analysis showed a tendency towards increased circulating CCAT1 expression in HER-2 positive patients.

3.2 DISCUSSION

Investigating the pattern of expression of circulating long non-coding RNA (lncRNA) CCAT1 in BC patients compared to healthy controls was the objective of this study. Based on our studies, breast cancer patients exhibit profoundly elevated levels of circulating CCAT1 in their peripheral blood. This result opens up the likelihood that CCAT1 is a non-invasive biomarker for diagnosis and can be actively engaged in the molecular mechanisms involved in breast cancer.

Compared with the control group of healthy individuals, breast cancer patients exhibited significantly greater relative expression of circulating CCAT1 based on quantitative analysis with RT-PCR. The statistical analysis resulted in a P-value of 0.0057, which reflected a very high level of significance and proved the hypothesis that the disease is associated with CCAT1 expression. A high degree of significance was suggested by the P-value of 0.0057 of the

statistical analysis, which favoured the hypothesis that the disease status is associated with CCAT1 expression. These findings are consistent with previous research that associated CCAT1 with various types of cancer as an oncogenic lncRNA. The function of LncRNA CCAT1 in promoting tumor growth, proliferation and metastasis in several malignancies, including colorectal, gastric, and hepatocellular carcinoma, has already been determined. The current findings complement previous reports and provide further insights into CCAT1's role in breast cancer, providing new ideas on its potential use in clinical diagnostics.

Breast cancer patients can actively secrete CCAT1 into the blood via exosomal delivery or other extracellular vesicle-mediated routes, as evidenced by the elevated levels of this molecule in their blood. This makes it a strong candidate for liquid biopsy methods, which are increasingly gaining traction in cancer diagnosis since they are non-invasive.

Moreover, the present study examined the correlation between HER2 receptor status—a significant molecular subtype in the classification of breast cancer—and CCAT1 expression. The clinical aggressiveness and distinctive molecular markers of HER2-positive tumors are well established. Based on our findings, HER2-positive patients generally had greater levels of CCAT1 expression compared to HER2-negative patients.

The trend towards higher expression in the HER2-positive group is noteworthy, although the variation in CCAT1 expression between the HER2-positive and HER2-negative groups failed to reach statistical significance ($P = 0.0635$). It opens the door to a correlation that requires further investigation with a larger sample size to validate these preliminary findings.

One of the limitations of this study is that the sample size of 10 patients per arm would have reduced the statistical power to detect subtle but important differences in CCAT1 expression. Larger cohorts in future studies would clarify the relationship between CCAT1 levels and specific subtypes of breast cancer, including hormone receptor and HER2 status.

Despite this limitation, our results emphasize the promise of the circulating lncRNA CCAT1 as a biomarker for breast cancer diagnosis. In contrast to conventional tissue biopsies, which are often uncomfortable and not always feasible in all clinical contexts, its overexpression in patient blood samples offers a convenient and less invasive option.

In addition, CCAT1 can be included as part of a panel of circulating biomarkers for enhancing sensitivity and specificity in detection of breast cancer, particularly for early-stage disease, if affirmed in larger populations. CCAT1 could enhance diagnostic accuracy and assist in directing treatment decisions in combination with other established markers.

There remains a great deal to discover regarding the biochemical mechanisms by which CCAT1 induces breast cancer. But as per previous research, CCAT1 presumably modulates gene expression through the alteration of chromatin, association with transcription factors, or functioning as a microRNA molecular sponge.

New targets for therapy can perhaps be identified by understanding the regulatory networks involving CCAT1 in breast cancer. Inhibition of CCAT1 could provide a new therapy strategy if shown to have a functional role in promoting tumor growth, especially in HER2-positive breast cancer where resistance to targeted agents is emerging as an increasing problem.

Briefly, the current research provides rigorous evidence that individuals with breast cancer possess significantly more of the lncRNA CCAT1 that circulates when compared to health individuals. Although additional research has to be performed to have total comprehension of how CCAT1 expression relates to HER2, the trend of what has already been discovered means that it does appear to be involved in some capacity with the subtyping of breast cancer.

As per our study, CCAT1 is a hopeful biomarker for early detection and description of breast cancer. In order to affirm the results and establish the clinical utility of CCAT1 in breast cancer screening and treatment as routine, greater and more heterogeneous cohorts are required in subsequent research.

CHAPTER 4

4.1 CONCLUSION

The present study provides strong evidence for the potential that the circulating long non-coding RNA (lncRNA) CCAT1 may be used as a new biomarker for breast cancer molecular profiling and diagnosis. With a statistically significant P-value of 0.0057, quantitative real-time PCR (qRT-PCR) analysis in the present study revealed that peripheral blood samples from breast cancer patients had a significantly higher expression level of CCAT1 compared to healthy controls. Due to this tight association, CCAT1 can serve as a non-invasive biomarker for diagnosing breast cancer at an early stage, which is essential for improving patient survival and prognosis.

Moreover, the investigation examined the relationship between HER2 receptor status—an important genetic classification in breast cancer and CCAT1 expression. The noted trend of higher levels of CCAT1 in HER2-positive individuals indicates a possible correlation, although the difference in CCAT1 expression between HER2-positive and HER2-negative patients did not reach statistical significance (P = 0.0635). This trend illustrates the promise of CCAT1 as a molecular classifier for breast cancer subtyping and as a diagnostic biomarker. To confirm this association and understand the underlying simple processes that make it happen, further research involving a larger sample size is needed.

Identification of CCAT1's diagnostic value through easily obtained blood samples is one of the strengths of the study and its suitability in routine clinic settings. The limitation of small sample size, specifically, could have been a contributing factor to the statistical power of relationship identification with molecular subtypes, but the study also acknowledges the limitations. The results thus need to be looked at cautiously and validated in larger and more diverse groups.

Despite these limitations, the findings contribute to the growing body of evidence showing the role of lncRNAs in cancer biology. Specifically, CCAT1 holds promise for early detection and potentially guiding treatment decisions, especially if follow-up studies establish its role in mechanisms of resistance or treatment responsiveness in specific subtypes of breast cancer.

In summary, circulating lncRNA CCAT1 is significantly upregulated in breast cancer patients and may represent an accessible, non-invasive biomarker for molecular classification and early detection. Its combination in a panel of biomarkers may enhance the accuracy of diagnosis and provide valuable information regarding disease course and prognosis. To address the full clinical potential of CCAT1 in breast cancer screening, subtyping, and treatment planning, additional research with larger sample sizes and mechanistic studies is warranted. Overexpression of CCAT1 aligns with previous reports that linked this long noncoding RNA to the carcinogenic processes of enhanced invasion, migration and growth in a range of malignancies, including colorectal, gastric and hepatocellular carcinoma. Its suitability as a target for liquid biopsy methods is also underlined by the potential that higher levels of circulating CCAT1 in breast cancer result from active secretion by cancer cells through exosomes or other extracellular vesicles. Such methods are extremely beneficial to patient care and the monitoring of long-term illness due to them being less invasive.

CHAPTER 5

5.1 APPLICATION OF CANCER BIOMARKERS IN FUTURE

Cancer biomarkers have the potential to revolutionize oncology in the future by facilitating early identification, accurate diagnosis, real-time monitoring and personalized treatment plans. These biological markers, which include circulating tumor cells and genetic alterations, have progressed beyond their conventional uses and are currently at the forefront of precision medicine. Biomarkers will become more precise, targeted and easily incorporated into clinical practice as technology develops **[34]**.

Early detection is one of the most promising uses of cancer biomarkers. Highly sensitive molecular markers like circulating tumor DNA (ctDNA), microRNAs, and metabolomic profiles can identify tumors at very early stages, often before symptoms appear **[35]**. Liquid biopsy technologies, in particular, enable non-invasive cancer diagnosis and monitoring through a simple blood draw, potentially improving outcomes through earlier, less invasive treatments **[36]**.

Further, biomarkers can also have extensive applications in targeted or personalized therapy. Physicians are able to tailor treatment plans when they identify different genetic and molecular changes in an individual's cancer **[37]**. For example, alterations of genes such as EGFR, KRAS, and BRAF are already being used to direct the treatment option for skin, lung, and colon cancers. More accurate targeted treatment is provided when new driver mutations and paths are discovered with a decrease in side effects and an improvement in efficacy **[38]**.

In addition, it is predicted that biomarkers for cancer will be crucial for monitoring treatment resistance and response. Treatment can be monitored for effectiveness in real time by the clinician by measuring biomarkers like ctDNA or cancer stem cell-specific markers (CSCs) serially **[39]**. The treatment approach can be rapidly changed if resistance occurs. Apart from enhancing the outcomes of treatment, this adaptive strategy avoids unnecessary exposure to ineffective therapies **[40]**.

Moreover, biomarkers are becoming into key resources in radiation oncology. Oncologists can adjust dosages and modalities for optimal results by employing certain markers that reflect a tumor's response to radiation. Toward enhanced patient outcomes and quality of life, parameters like tumor hypoxia, glucose uptake and EGFR expression are being researched already to tailor radiation treatment regimens **[41]**.

In addition, enhancing the specificity and reliability of biomarkers will be the central concern of research in the future, especially where genetic factors and multiple exposures interact in complex situations. Validation in a range of populations and standardization of the testing methodologies as well as more robust quantification strategies will necessitate the conversion of biomarker research into everyday clinical tools **[42]**.

With advancements in biomarker research, hundreds of molecular points can be tested simultaneously due to integrative platforms and advanced bioinformatics. With this, overall tumor signatures will be created that will assist with diagnosis, therapy and extended care. Such detailed molecular characterization will form the basis for genuinely personalized cancer therapy in the future **[43]**.

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FIGURE REFRENCES

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TABLE REFRENCE

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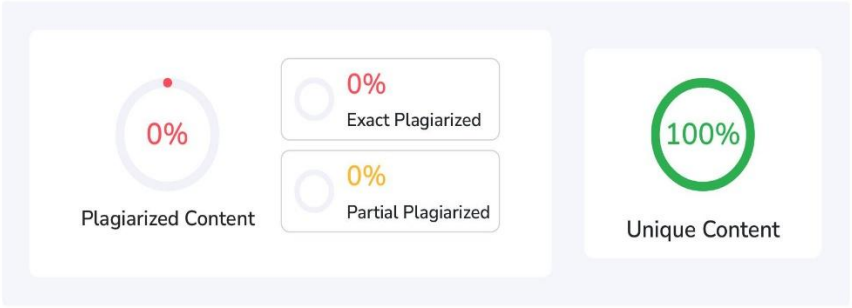

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ABSTRACT

Long non-coding RNAs (lncRNAs) have emerged as critical regulators in various biological processes, including cancer development and progression. Among them, Colon Cancer Associated Transcript 1 (CCAT1) is a well-characterized lncRNA that has been implicated in several malignancies due to its role in gene expression regulation, cellular proliferation, migration, and metastasis. The precise contribution of CCAT1 to breast cancer development and progression remains to be fully elucidated. This study aims to investigate the expression pattern of CCAT1 in breast cancer tissues and its correlation with clinic-pathological parameters in breast cancer patients to uncover its potential as blood-based predictive and prognostic biomarker.

Ten women who had been diagnosed with breast cancer and ten healthy individuals had peripheral blood samples taken for comparison. The blood was then processed, and RBC lysis buffer was used to extract the white blood cells (WBCs). Total RNA was then extracted from WBCs using the TRIzol reagent. Then, using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) with GAPDH as an endogenous reference and CCAT1 as the target gene, the expression level of lncRNA CCAT1 in breast cancer patients was evaluated in relation to healthy controls.

The expression levels of the lncRNA CCAT1 in the circulating white blood cells (WBCs) of patients with breast cancer were significantly different from those of healthy controls, according to our findings. The obtained data indicate that lncRNA CCAT1 expression levels may act as an informative biomarker for breast cancer diagnosis. Farther investigation with a larger cohort is needed to determine the potential of lncRNA CCAT1 as a predictive and prognostic biomarker in the breast cancer patients.

KEYWORDS:

Breast cancer, Long non-coding RNA (lncRNA), CCAT1, qRT-PCR, Circulating blood-based Biomarker, Gene expression, Liquid biopsy, Cancer diagnosis

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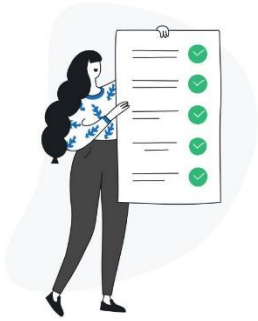

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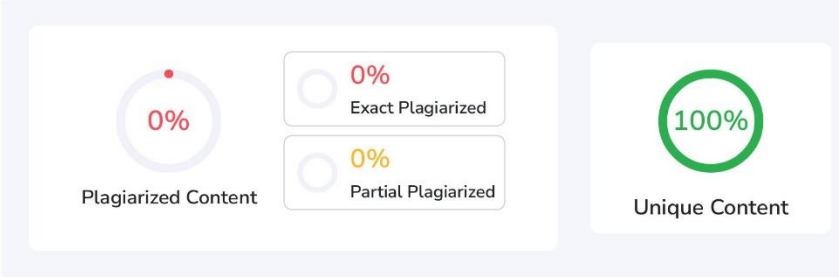

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CHAPTER 1

1.1 CANCER

Cancer is a complex disease that develops from abnormal cells that grow and spread in an uncontrolled manner. Its appearance is influenced by a mix of environmental, occupational, lifestyle and genetic factors. Historical research also has shown that things such as occupational hazards, synthetic chemicals and infectious agents pose a major cancer risk. [1]

As the research continued, the role of infectious agents such as human papillomavirus (HPV) and hepatitis viruses (HBV and HCV) became clearer; this expanded the understanding of cancer beyond toxic exposure. Recent studies have strengthened associations between a variety of environmental and occupational exposures and specific cancers. Air pollution, for instance, has been linked to an increased risk of developing lung cancer. Exposure to pesticides has been linked to increased risks of breast and prostate and other cancers. [2]

Decreased exposure to known carcinogens is a proven mechanism to reduce cancer incidence, so the progressive reductions in male lung cancer were attributed to decreased tobacco usage..

1.2 BREAST CANCER

With 1.2 million new cases and 500,000 fatalities annually, breast cancer is the most common cancer in women worldwide and continues to be a major cause of cancer-related mortality. Though they are more prevalent in developed countries, these spreads are becoming more frequent in developing countries. [4]

According to 2017 statistics, breast cancer was estimated to account 30% (252,710) of all new cases of cancer among women in the United States. [4]

The disease primarily occurs in postmenopausal women, and a substantial proportion of cases are hormone-responsive. Hormonal dependency, as well as genetic and environmental variables, contribute to the initiation and progression of breast cancer. [5]

This cancer can spread to distant organs, such as the bones, liver, lungs and brain, making it more challenging to treat. [4]

Breast cancer screening is usually done via mammography whereas systemic therapies such as tamoxifen reduce the risk of mortality. Moreover, over the past decade, Magnetic Resonance Imaging (MRI), which provides higher sensitivity, has been used and studied more extensively. [6]

With 1.2 million new cases and 500,000 fatalities annually, breast cancer is the most common cancer in women worldwide and continues to be a major cause of cancer-related mortality. Although more common in developed countries, such outbreaks are increasingly occurring in developing nations as well. [7]

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1.3 TYPES OF BREAST CANCER

In discussing the risk of breast cancer associated with benign breast disease (BBD), McDivitt and colleagues' comment that many BBDs (e.g. hyperplasia and atypia) can increase the risk of the subsequent development of malignancy substantially. Women with benign tumors called fibroadenomas are similarly at an increased risk. This study highlights the importance of monitoring benign conditions to prevent any progression [8], though the study isn't designed to address subtypes such as Luminal A, Luminal B, HER2-positive, or Triple-Negative breast cancer.

Colleoni and co-workers focus on luminal breast cancer subtypes, noting that Luminal A tumours, which have hormone receptors but don't overexpress HER2, frequently present with a lower proliferation rate and a better prognosis, and are often treated with endocrine therapies. In contrast, Luminal B tumors tend to be more aggressive, with the potential for HER2 amplification, and an overall worse prognosis. Their studies underscore the need for precise identification of subtypes to tailor treatments appropriately [9].

HER2 overexpression defines HER2-positive breast cancer, which is aggressive but generally well - treated using drugs such as trastuzumab. On the other hand, Triple-Negative Breast Cancer (TNBC) which is characterized by the absence of estrogen, progesterone and HER2 receptors, is a highly aggressive subtype with few treatments available aside from chemotherapy. The importance of histological and molecular assessments is reiterated by Reis-Filho and Lakhani for an understanding of breast cancer complexity and targeted treatment determination [10].

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I) STRUCTURAL BASED:
i) Ductal Carcinoma In-Situ (DCIS): DCIS is a kind of breast cancer in which the aberrant cells remain inside the milk ducts rather than spreading to neighbouring tissues.[11]

ii) Invasive Ductal Carcinoma (IDC): The most prevalent form of breast cancer is IDC. It occurs when cancer cells begin to infiltrate the surrounding breast tissue after rupturing the duct walls. Tumor size, involvement of lymph nodes, and the state of specific biomarkers are some of the variables that can affect the prognosis. [11]

iii) Invasive Lobular Carcinoma (ILC): ILC begins in the milk-producing lobules and has the potential to spread to neighbouring tissues. Despite being less frequent than IDC, it is frequently discovered later because of its more diffuse growth pattern, which makes detection more difficult. [12]

II) CLINICOPATHALOGICAL PARAMETERS-BASED:
A tumor size, grade, lymph node involvement, and immunohistochemical (IHC) markers are all considered when categorizing breast cancer. Higher grades frequently indicate more aggressive behavior, while larger tumors typically indicate more advanced stages. Understanding nodal involvement aids in determining the prognosis and the extent of the cancer's spread. Progesterone receptor (PR), estrogen receptor (ER), and HER2 status are examples of IHC indicators that are crucial for guiding therapy choices and predicting the effectiveness of treatments [12].

III) HORMONE RECEPTORS-BASED:
Breast cancer subtypes include HER2-enriched, Triple-Negative, Luminal A, and Luminal B biomarkers. Luminal A (ER+, PR+, HER2-) responds well to hormone therapy, develops slowly, and is unlikely to recur. More aggressive and possibly HER2+, luminal B frequently necessitates a mix of hormone and HER2-targeted therapies. Although HER2-enriched (ER-, PR-, HER2+) cancer spreads swiftly, it can be successfully treated with trastuzumab and other HER2-targeted medications. Although immunotherapy is beginning to show promise, Triple-Negative (ER-, PR-, HER2-) is especially aggressive and has fewer options for targeted therapy. [13]

IV) IMMUNE CHEECKPOINT REGULATORS BASED:
In the fight against breast cancer, especially the more aggressive types like triple-negative breast cancer (TNBC), the immune system plays a critical role. PD-L1, an immune checkpoint protein that enables cancer cells to evade the body's immune responses, is a crucial participant in this process. Pembrolizumab and other immune checkpoint inhibitors have been shown to have a positive effect on

tumors that express PD-L1. For PD-L1-positive cancers, this immunotherapy strategy holds out a lot of promise for improving treatment results and giving patients new hope. [14]

1.5 RISK FACTORS OF BREAST CANCER

Breast cancer is a multifaceted illness that arises from a sequence of events involving various cell types and genetic alterations. Usually beginning with ductal hyperproliferation, it can develop into tumors due to the tumor microenvironment, which includes elements such as aberrant DNA methylation patterns and inflammatory macrophages. The development of tumors, their resistance to therapy, and the likelihood of recurrence are all significantly influenced by breast cancer stem cells (bCSCs). Furthermore, it is known that cancer progresses due to mutations in key genes like EGFR, HER2, BRCA1, and BRCA2. The cancer stem cell theory, which holds that all tumor subtypes originate from the same stem cells, and the stochastic theory, which contends that any breast cell can accumulate random mutations that cause cancer, are the two main theories regarding the genesis of breast cancer [15].

The chance of getting breast cancer can be raised by a number of risk factors. A major contributing factor is age; women over 40 account for the majority of cases. Risk can be significantly increased by a family history of the condition, especially if the BRCA1 and BRCA2 genes are mutated. Early menstruation, a delayed menopause, and a later first pregnancy are examples of reproductive factors that also come into play. Hormonal factors that increase risk include hormone replacement therapy (HRT) and increased estrogen levels from birth control pills. The risk is also increased by lifestyle choices such as smoking, eating a high-fat diet, drinking alcohol, being overweight and not exercising enough [16].
To help reduce the risk of breast cancer and improve survival rates, preventive measures like genetic screening, making healthier lifestyle choices and early detection through mammograms are essential [17].

1.6 DIAGNOSIS

A combination of screening procedures, imaging methods, biopsies, and laboratory testing is usually used to diagnose breast cancer. Although mammography is the most common screening technique, MRI and ultrasound are also useful tests, particularly for individuals who are more susceptible. To determine whether a suspicious lump is cancerous, a doctor will typically perform a biopsy. This could be a core needle biopsy, a fine-needle aspiration, or even a surgical biopsy [18]. The treatment plan is then shaped by laboratory tests that examine genetic mutations like BRCA1 and BRCA2, hormone receptors (like ER/PR), and HER2 status. Physicians may use bone scans, CT scans, PET scans, or lymph node biopsies to determine whether the cancer has spread. Survival rates can be considerably increased by detecting it early through routine screenings and follow-ups [19].

1.7 LONG NON-CODING RNA

Long non-coding RNAs, or lncRNAs, are interesting RNA molecules measuring more than 200 nucleotides. Although they do not code for proteins, they perform crucial functions in gene regulation, chromatin remodelling and maintenance of diverse cellular processes. These molecules are transcribed by RNA Polymerases I, II and III and even may originate from processed introns. In contrast to their protein-coding relatives, lncRNAs are rapidly evolving, are specific to various cell types and are major contributors to cell differentiation, development and physiology [14]
LncRNAs interact with chromatin-modifying complexes, which assists in shaping gene expression and genome organization. In addition, they play functions in the cytoplasm, which affects translation, metabolism and signaling pathways. Most lncRNAs possess modular structures and repeat sequences, which increase their functionality. Further, they are linked to different disease mechanisms, such as cancer development and neurological disorders. [15]
Despite their importance to various biological functions, it can be very challenging to classify and annotate lncRNAs because they have very complex relationships with other genes. It is only going to advance in the future our knowledge about their biological functions, structural properties and therapeutic possibilities in the treatment of diseases. [16]

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It occurs when cancer cells begin to infiltrate the surrounding breast tissue after rupturing the duct walls.
https://www.nationalbreastcancer.org/invasive-ductal-carcinoma

To determine whether a suspicious lump is cancerous, a doctor will typically perform a biopsy.
https://www.radiologyinfo.org/en/info/biopgen

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1.8 BIOMARKERS

The term "biomarker" is derived from the term "biological marker" and signifies measurable medical signs that are detectable and can be measured (Strimbu & Tavel, 2010). Based on the International Programme on Chemical Safety, managed by the World Health Organization (WHO) together with the United Nations and the International Labor Organization, a biomarker refers to any quantifiable substance, structure or process in the body which has the potential to influence or forecast the incidence or the consequences of disease. [17].

Biomarkers may be proteins such as enzymes and receptors, nucleic acids such as microRNAs and non-coding RNAs, antibodies and peptides. They may also reflect complicated biological alterations, such as gene expression and proteomic or metabolomic patterns. Since biomarkers are usually present in biological fluids such as blood (serum, plasma), excretions, and secretions (urine, stool, sputum, nipple discharge), they can be measured non-invasively and serially. However, a biopsy or imaging will be necessary to accurately assess these biomarkers [18].

Hereditary genetic biomarkers can be classified as either somatic- mutations acquired in tumor DNA or germline which are inherited and detectable in DNA from sources like blood, sputum, or buccal cells. Understanding how diseases are identified, how they develop, and how well therapies are working depends on these markers. [19]

1.9 RNA BASED BIOMARKERS

RNA-derived biomarkers are increasingly popular for their significant contribution to the diagnosis, prediction and monitoring of diseases. Messenger RNAs (mRNAs) and microRNAs (miRNAs) are useful indicators in the case of colorectal cancer (CRC). Both provide useful information regarding the formation and growth of tumors. Through RNA expression patterns, researchers can identify molecular alterations linked to CRC, which can be used to detect the disease early through non-invasive techniques such as stool and blood tests. [20]

RNA biomarkers also have an important role in predicting the course of disease and the likelihood of recurrence, opening the door to individualized treatment strategies. For example, changes in miRNAs may influence how patients will respond to chemotherapy, particularly with fluorouracil-based therapies, guiding more effective therapy choices. [21]

There remain, however, some challenges to be overcome, like genetic heterogeneity, problems in RNA stability and the need for standardized protocols. [23] Circulating tumor RNAs and stool-based miRNAs notwithstanding these challenges are very promising in increasing CRC screening.

One such region of special concern is the long non-coding RNA (lncRNA) CCAT1, which may act as a non-invasive biomarker associated with CRC development, gene expression and oncogenic pathways. CCAT1 has implications beyond CRC, being implicated in gastric, liver and breast cancers highlighting its role as a target for future therapies. [24]

1.10 TYPES OF RNA BASED BIOMARKERS

i) MicroRNAs (miRNAs) are small, non-coding RNAs that are involved in an important regulation of gene expression post-transcriptionally by binding to the 3' UTR of target mRNA, modulating processes such as proliferation of cells, differentiation and apoptosis as cancer activating or cancer blocking genes. [25]

ii) Long Non-Coding RNAs (lncRNAs), more than 200 nucleotides, and play significant roles in the regulation of genes and remodeling chromatin. Certain lncRNAs, such as CRCAL-3 and CRCAL-4, were discovered to be dysregulated in colorectal cancer (CRC) and influence the regulation of the cell cycle. [20]

iii) Circular RNAs (circRNAs) stable RNAs form covalently closed loops and acts as miRNA sponges, managing gene transcription and assisting protein translation. [26]

iv) Small Nucleolar RNAs (snoRNAs) are also significant; they are involved in the maturation of ribosomal RNA and are associated with processes such as alternative splicing and silencing of genes. [28]

v) Piwi-Interacting RNAs (piRNAs) are short RNAs associate with Piwi proteins and participate in silencing transposons, cell proliferation and cancer development. [27]

vi) Messenger RNAs (mRNAs) are of prime type and their expression patterns have the potential to give important clues to disease pathology. PAM50 and other multi-gene panels are used to categorize and forecast the prognosis of breast cancer. [27]

vii) Extracellular RNAs (exRNAs), which are present in biofluids like saliva and serum. These are promising non-invasive biomarkers since they include miRNAs and circRNAs and are frequently maintained by vesicle structures or RNA-binding proteins. [28]

1.11 CCAT1 AND ITS ROLE IN BREAST CANCER

Long non-coding RNA CCAT1 has a significant role in the progression, metastasis, and prognosis of breast cancer. Studies suggest that CCAT1 is strongly upregulated in breast cancer tissues, particularly in breast cancer stem cells (BCSCs) and is associated with worse prognosis for patients. [29]

CCAT1 promotes cancer cell proliferation, invasion and migration through the activation of the WNT/β-catenin signaling pathway. TCF4 is upregulated and β-catenin is translocated to the nucleus as a result of interaction with Annexin A2 (ANXA2) and certain microRNAs (miR-204/211 and miR-148a/152). This activation augments cancer stemness, and this is implicated in tumor metastasis and recurrence. [30]

For triple-negative breast cancer (TNBC), CCAT1 promotes tumor development through suppressing miR-218/ZFX signaling. If CCAT1 is knocked down, there's upregulation of miR-218 that suppresses the ZFX oncogene, thereby reducing TNBC cell growth, migration, and invasion. This indicates that CCAT1 functions as an oncogene in TNBC by targeting pathways that typically inhibit tumors. [31]

Furthermore, CCAT1 also presents as a promising prognostic biomarker. Its overexpression is associated with increased tumor stages, lymph node metastases and poorer survival rates in breast cancer patients [32]. Due to its primary role in the progression of cancer, CCAT1 may prove to be an effective therapeutic target against breast cancer. [33]

CHAPTER 2

MATERIALS & METHODOLOGY

BLOOD PROCESSING

2.1 Sample: Eleven breast cancer patients' peripheral blood samples and ten healthy control peripheral blood samples were obtained. (See Table 2)

2.2 Equipment & Instruments

- Pipettes & Pipette Tips (for precise liquid handling)
- Vortex Mixer (for homogenization)
- Microcentrifuge Tubes (MCTs) (sterile, for sample collection)
- Applied Biosystems thermocycler,
- Nanodrop Lite
- PCR hood
- CFX96 RT-qPCR
- Eppendorf centrifuge (capable of cooling to 4°C and high-speed spins)
- Vortex (for homogenization)

- Heating block
- pH meter
- Micropipettes

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Furthermore, CCAT1 also presents as a promising prognostic biomarker.
https://pmc.ncbi.nlm.nih.gov/articles/PMC5955114

- **Eppendorf centrifuge (capable of cooling to 4°C and high-speed spins)**
https://www.eppendorf.com/us-en/Products/Centrifugation/Microcentrifuges/Centrifuge-5425-5425R-p-PF-934144

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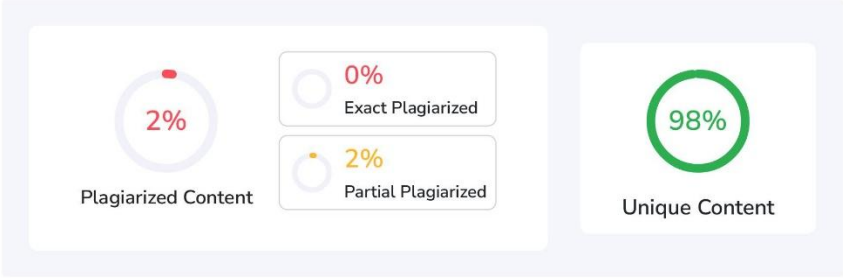

Total Words: 795 **Total Characters:** 4852 **Plagiarized Sentences:** 0.92 **Unique Sentences:** 45.08 (98%)

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2.3 Reagents & Buffers

- RBC Lysis Buffer (for removal of red blood cell)
- Phosphate-Buffered Saline (PBS) (for cleaning the pellet)
- TRIZOL Reagent (for nucleic acid preservation)
- Sigma analytical grade chloroform
- Sigma analytical grade isopropanol
- 75% ethanol
- NFH20
- 5x QuantiScriptRT Buffer
- RT Primer Mix,
- gDNA Wipeout Buffer 7x
- Quantiscript Reverse Transcriptase
- 2x QuantiTect SYBR Green
- Forward and Reverse primers for MALAT1
- Forward and Reverse primers for GAPDH

2.4 Miscellaneous:

- EDTA Tubes
- PCR tubes
- MCTs
- Cryogenic Storage (-80°C Freezer) (for long-term preservation of sample)
- Labels & Markers (for proper sample identification)

2.5 WBC ISOLATION PROTOCOL

This protocol is performed to efficiently separate and preserve different blood components for downstream molecular analysis. Plasma is isolated for biochemical and proteomic studies, while cellular components are processed for nucleic acid extraction. The use of RBC lysis buffer, PBS washing, and TRIZOL reagent ensures high-quality isolation of nucleic acids for genetic and transcriptomic research.

Blood processing for Breast Carcinoma patients and healthy controls:

A. Blood samples were collected from eleven Breast carcinoma patients and ten healthy controls in EDTA Tubes.

B. The blood samples were centrifuged at 2000g for 10 minutes at 4°C. After centrifugation, the components of blood (Red Blood Cells, White Blood Cells, and platelets) precipitate to the bottom while the plasma remains at the top of the EDTA tubes.

C. The plasma was then separated carefully from the EDTA Tubes by pipetting. This whole process was conducted on ice to prevent the degradation of RNAs present in the samples.

D. After the separation of plasma, RBC lysis buffer was added to the EDTA tubes and incubated for 20 minutes. RBC lysis buffer is effective for lysing nucleated red blood cells.

F. After discarding the supernatant, WBCs were washed with RBC lysis buffer 2-3 times to eliminate the presence of extra RBCs in the samples.
Plasma was put into sterile MCTs → Stored at -80°C.

RNA extraction from WBCs by TRIzol reagent:
TRIzol reagent was used to extract RNA from the obtained WBCs
A. White Blood Cells obtained from blood processing were suspended in 1 ml of TRIzol.
B. For lysis, 0.2 ml chloroform was added per 1 ml of TRIzol Reagent, then the mixture was thoroughly homogenized by pipetting up and down.
C. Incubated for 10-15 minutes.
D. The sample was centrifuged for 30 minutes at 12,000×g at 4°C. The combination separates into a colorless upper aqueous phase, an interphase, and a lower red phenol-chloroform.
E. The aqueous phase containing the RNA was transferred to a new tube by angling the tube at 45° and pipetting the solution out.
F. For lysis, 0.5 ml of isopropanol was added to the aqueous phase per 1 ml of TRIzol Reagent.
G. Incubated for 1 hour at 4° C.
H. The sample was centrifuged for 30 minutes at 12000×g at 4° C. Total RNA precipitate formed a white gel-like pellet at the bottom of the tube.
L. The supernatant was discarded by using a micropipette.
M. The RNA pellet was dried in air or vacuum for 5-10 minutes.
N. The pellet was resuspended in 20-50 µl of RNase-free water (warm) by pipetting up and down.
O. Incubated in a water bath or heat block at 55-60°C for 10-15 minutes.
P. Total RNA was measured at nanodrop/qubit
Q. Storage for Future Analysis at -80°C

2.6 RNA Quantification
STEP 1:
Qubit Fluorometer was used with RNA High Sensitivity Kit
↓
If RNA concentration was low → Concentrated with vacuum concentrator
↓
Re-quantify RNA was done using Qubit 4/nanodrop

cDNA Preparation with QuantiTect Reverse Transcription:
A. The Quantitect RT Kit was used to make the cDNA.

TABLE 2: cDNA Reaction Master Mix
Change required
10 Healthy Control's sample
10 Breast Cancer patients' samples (µL) Total (µL)
Quantiscript Reverse Transcription 1x2 = 2 1x2 = 2 4
RT Buffer 5X 4x2 = 8 4x2 = 8 16
RT Primermix 1x2 = 2 1x2 = 2 4
Total Volume 12 12 24

D. Incubate for 2 min at 42℃. Then place immediately on ice.
E. Prepare the reverse-transcription reaction master mix according to the manufacturer's instructions (Table 3).

TABLE 3: RT-qPCR Reaction Mix
Change Required
CCAT1 GAPDH
4X2 (Duplicate)+1NTC = 9 wells 4X2 (Duplicate)+1NTC = 9 wells
SYBR Green 5x9 = 45 5x9 = 45
Forward Primer 1x9 = 9 1x9 = 9
Reverse Primer 1x9 = 9 1x9 = 9
NF-H20 2x9 = 18 2x9 = 18
Total 9 µL /well 9 µL /well

I. Quantitative real-time PCR.

2.5 Real-Time PCR (qPCR)
After cDNA preparation, the Quantitect SYBRGreen PCR kit was used to perform RT-qPCR. The following are the steps involved in the procedure:
A. Make a reaction mix according to the instructions (Table 4).

Plagiarized Sources

Quantiscript Reverse Transcription 1x2 = 2 1x2 = 2 4
https://www.avantorsciences.com/us/en/product/18667760/quantitect-rev-transcription-kit-qiagen

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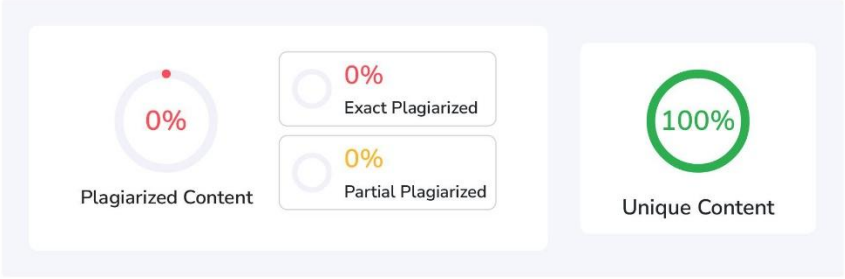

Total Words: 922 **Total Characters:** 6335 **Plagiarized Sentences:** 0 **Unique Sentences:** 40 (100%)

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D. Set up the real-time cycler in Figure 10

E. The target gene's relative expression levels were measured using the endogenous house-keeping gene GAPDH as an internal control.
qPCR was done using SYBR Green or probe-based master mix.

CHAPTER 3
3.1 qRT-PCR RESULTS
Quantitative Real Time-PCR analysis revealed that the expression of lncRNA CCAT1 decreases significantly in the white blood cells (WBCs) of the eleven Breast cancer patients compared to that of the ten healthy control samples. The red colour curves are for endogenous control (GAPDH) and the blue colour curves are for lncRNA CCAT1.

The expression levels of lnRNA CCAT1 in the peripheral blood of the 10 breast cancer patients and 10 healthy individuals as control was analysed by performing RT-PCR. It was observed in the study that the circulating lnRNA CCAT1 is overexpressed in samples of breast cancer patients when compared to normal healthy controls. Through the statistical analysis it was found that significantly (P = 0.0057) circulating CCAT1 levels are upregulated in BC patients.
Circulating CCAT1 is upregulated in HER2- positive patients as compared to HER2- negative patient

Further, it was concluded that circulating CCAT1 expression levels has the potential to be used as biomarkers to identify HER-2 positive and HER-2 negative breast cancer patients. After performing RT-PCR and calculating the relative quantification, the statistical analysis was performed, which showed the result that was not statistically significant (P = 0.0635). The statistical analysis showed a tendency towards increased circulating CCAT1 expression in HER-2 positive patients.

3.2 DISCUSSION
Investigating the pattern of expression of circulating long non-coding RNA (lncRNA) CCAT1 in BC patients compared to healthy controls was the objective of this study. Based on our studies, breast cancer patients exhibit profoundly elevated levels of circulating CCAT1 in their peripheral blood. This result opens up the likelihood that CCAT1 is a non-invasive biomarker for diagnosis and can be actively engaged in the molecular mechanisms involved in breast cancer.
Compared with the control group of healthy individuals, breast cancer patients exhibited significantly greater relative expression of circulating CCAT1 based on quantitative analysis with RT-PCR. The statistical analysis resulted in a P-value of 0.0057, which reflected a very high level of significance and

proved the hypothesis that the disease is associated with CCAT1 expression. A high degree of significance was suggested by the P-value of 0.0057 of the statistical analysis, which favored the hypothesis that the disease status is associated with CCAT1 expression. These findings are consistent with previous research that associated CCAT1 with various types of cancer as an oncogenic lncRNA. The function of LncRNA CCAT1 in promoting tumor growth, proliferation, and metastasis in several malignancies, including colorectal, gastric, and hepatocellular carcinoma, has already been determined. The current findings complement previous reports and provide further insights into CCAT1's role in breast cancer, providing new ideas on its potential use in clinical diagnostics.

Breast cancer patients can actively secrete CCAT1 into the blood via exosomal delivery or other extracellular vesicle-mediated routes, as evidenced by the elevated levels of this molecule in their blood. This characteristic renders it a potential candidate for liquid biopsy methods, which are increasingly gaining traction in cancer diagnosis since they are non-invasive.

Moreover, the present study examined the correlation between HER2 receptor status—a significant molecular subtype in the classification of breast cancer—and CCAT1 expression. The clinical aggressiveness and distinctive molecular markers of HER2-positive tumors are well established. Based on our findings, HER2-positive patients generally had greater levels of CCAT1 expression compared to HER2-negative patients.

The trend towards higher expression in the HER2-positive group is noteworthy, although the variation in CCAT1 expression between the HER2-positive and HER2-negative groups failed to reach statistical significance ($P = 0.0635$). It opens the door to a correlation that requires further investigation with a larger sample size to validate these preliminary findings.

One of the limitations of this study is that the sample size of 10 patients per arm would have reduced the statistical power to detect subtle but important differences in CCAT1 expression. Larger cohorts in future studies would clarify the relationship between CCAT1 levels and specific subtypes of breast cancer, including hormone receptor and HER2 status.

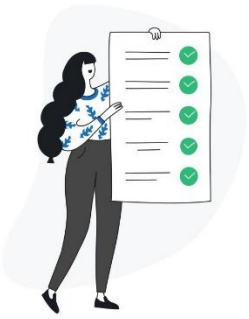
Despite this limitation, our results emphasize the promise of the circulating lncRNA CCAT1 as a biomarker for breast cancer diagnosis. In contrast to conventional tissue biopsies, which are often uncomfortable and not always feasible in all clinical contexts, its overexpression in patient blood samples offers a convenient and less invasive option.

In addition, CCAT1 could enhance diagnostic accuracy and assist in directing treatment decisions in combination with other established markers.

There remains a great deal to discover regarding the biochemical mechanisms by which CCAT1 induces breast cancer. But as per previous research, CCAT1 presumably modulates gene expression through the alteration of chromatin, association with transcription factors, or functioning as a microRNA molecular sponge.

New targets for therapy can perhaps be identified by understanding the regulatory networks involving CCAT1 in breast cancer. Inhibition of CCAT1 could provide a new therapy strategy if shown to have a functional role in promoting tumor growth, especially in HER2-positive breast cancer where resistance to targeted agents is emerging as an increasing problem.

Briefly, the current research provides rigorous evidence that individuals with breast cancer possess significantly more of the lncRNA CCAT1 that circulates when compared to health individuals. Although additional research has to be performed to have total comprehension of how CCAT1 expression relates to HER2, the trend of what has already been discovered means that it does appear to be involved in some capacity with the subtyping of breast cancer.



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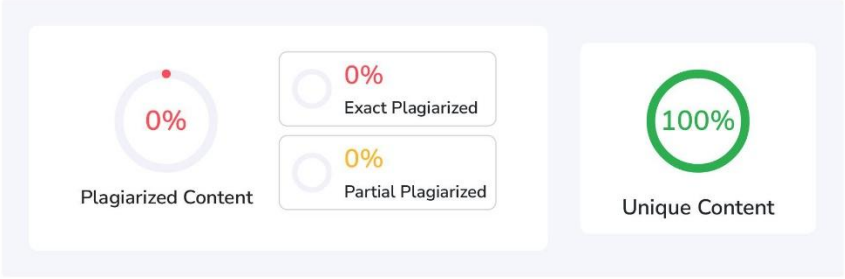

Total Words: 953 **Total Characters:** 6745 **Plagiarized Sentences:** 0 **Unique Sentences:** 40 (100%)

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CHAPTER 4

4.1 CONCLUSION

The present study provides strong evidence for the potential that the circulating long non-coding RNA (lncRNA) CCAT1 may be used as a new biomarker for breast cancer molecular profiling and diagnosis. With a statistically significant P-value of 0.0057, quantitative real-time PCR (qRT-PCR) analysis in the present study revealed that peripheral blood samples from breast cancer patients had a significantly higher expression level of CCAT1 compared to healthy controls. Due to this tight association, CCAT1 can serve as a non-invasive biomarker for diagnosing breast cancer at an early stage, which is essential for improving patient survival and prognosis.

Moreover, the investigation examined the relationship between HER2 receptor status—an important genetic classification in breast cancer—and CCAT1 expression. The noted trend of higher levels of CCAT1 in HER2-positive individuals indicates a possible correlation, although the difference in CCAT1 expression between HER2-positive and HER2-negative patients did not reach statistical significance (P = 0.0635).To confirm this association and understand the underlying simple processes that make it happen, further research involving a larger sample size is needed.

Identification of CCAT1's diagnostic value through easily obtained blood samples is one of the strengths of the study and its suitability in routine clinic settings. The limitation of small sample size, specifically, could have been a contributing factor to the statistical power of relationship identification with molecular subtypes, but the study also acknowledges the limitations. The results thus need to be looked at cautiously and validated in larger and more diverse groups.

Despite these limitations, the findings contribute to the growing body of evidence showing the role of lncRNAs in cancer biology. Specifically, CCAT1 holds promise for early detection and potentially guiding treatment decisions, especially if follow-up studies establish its role in mechanisms of resistance or treatment responsiveness in specific subtypes of breast cancer.

In summary, circulating lncRNA CCAT1 is significantly upregulated in breast cancer patients and may represent an accessible, non-invasive biomarker for molecular classification and early detection. Its combination in a panel of biomarkers may enhance the accuracy of diagnosis and provide valuable information regarding disease course and prognosis. To address the full clinical potential of CCAT1 in breast cancer screening, subtyping, and treatment planning, additional research with larger sample sizes and mechanistic studies is warranted. Overexpression of CCAT1 aligns with previous reports that linked this long noncoding RNA to the carcinogenic processes of enhanced invasion, migration, and growth in a range of malignancies, including colorectal, gastric and hepatocellular carcinoma. Its suitability as a target for liquid biopsy methods is also underlined by the potential that higher levels of circulating CCAT1 in breast cancer result from active secretion by cancer cells through exosomes or other extracellular vesicles. Such methods are extremely beneficial to patient care and the monitoring of long-term illness due to them being less invasive.

CHAPTER 5

5.1 APPLICATION OF CANCER BIOMARKERS IN FUTURE

Cancer biomarkers have the potential to revolutionize oncology in the future by facilitating early identification, accurate diagnosis, real-time monitoring, and individualized treatment plans. These biological markers, which include circulating tumor cells and genetic alterations, have progressed beyond their conventional uses and are currently at the forefront of precision medicine. Biomarkers will become more precise, targeted, and easily incorporated into clinical practice as technology develops [34].

Early detection is perhaps the most promising application of cancer biomarkers. Circulating tumor DNA (ctDNA), microRNAs, and metabolomic profiles are some of the highly sensitive molecular markers that can be utilized to detect tumors at very early stages, often before symptoms develop [35]. By facilitating early and potentially less invasive treatments, this early detection may significantly enhance patient outcomes. Most notably is the innovation of liquid biopsy technologies, which transform cancer monitoring and management through the ability to diagnose and monitor tumors non-invasively by a simple blood draw [36].

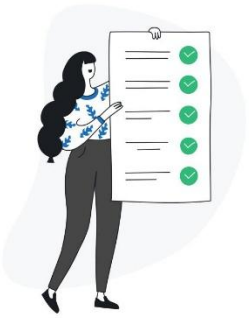
Further, biomarkers can also have extensive applications in targeted or personalized therapy. Physicians are able to tailor treatment plans when they identify different genetic and molecular changes in an individual's cancer [37]. For example, alterations of genes such as EGFR, KRAS, and BRAF are already being used to direct the treatment option for skin, lung, and colon cancers. More accurate targeted treatment is provided when new driver mutations and paths are discovered with a decrease in side effects and an improvement in efficacy [38].

In addition, it is predicted that biomarkers for cancer will be crucial for monitoring treatment resistance and response. Treatment can be monitored for effectiveness in real time by the clinician by measuring biomarkers like ctDNA or cancer stem cell-specific markers (CSCs) serially [39]. The treatment approach can be rapidly changed if resistance occurs. Apart from enhancing the outcomes of treatment, this adaptive strategy avoids unnecessary exposure to ineffective therapies [40].

Moreover, biomarkers are becoming into key resources in radiation oncology. Oncologists can adjust dosages and modalities for optimal results by employing certain markers that reflect a tumor's response to radiation. Toward enhanced patient outcomes and quality of life, parameters like tumor hypoxia, glucose uptake, and EGFR expression are being researched already to tailor radiation treatment regimens [41].

In addition, enhancing the specificity and reliability of biomarkers will be the central concern of research in the future, especially where genetic factors and multiple exposures interact in complex situations. Validation in a range of populations and standardization of the testing methodologies as well as more robust quantification strategies will necessitate the conversion of biomarker research into everyday clinical tools [42].

With advancements in biomarker research, hundreds of molecular points can be tested simultaneously due to integrative platforms and advanced bioinformatics. With this, overall tumor signatures will be created that will assist with diagnosis, therapy, and extended care. Such detailed molecular characterization will form the basis for genuinely personalized cancer therapy in the future [43].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